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A R Tall, … , D M Small, R S Lees

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Research Article

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Interaction of Collagen with the Lipids of Tendon Xanthomata

ALAN R. TALL, DONALD M. SMALL, and ROBERT S. LEES, *Biophysics Section, Division of Medicine, Boston University, School of Medicine, Boston, Massachusetts 02118, and the Arteriosclerosis Center, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*

ABSTRACT To determine the physical state of lipids in tendon xanthomata, six specimens surgically removed from three patients with familial hypercholesterolemia were studied by microscopy, calorimetry, and X-ray diffraction. The major constituents of the xanthomata were lipid (33% of dry weight) and collagen (24% of dry weight). The principal lipids were cholesterol ester and cholesterol. Light microscopy and thin-section electron microscopy showed occasional clusters of foam cells separated by masses of extracellular collagen. Polarized light microscopy of fresh, minced tissue showed rare droplets of free cholesterol ester. When heated, the tissue shrank abruptly at ≅70°C and, consequently, a large amount of cholesterol ester was released. Scanning calorimetry of fresh pieces of xanthoma showed a single, broad, reversible liquid crystalline transition of cholesterol ester with peak temperature from 32 to 38°C. The enthalpy (0.71±0.07 cal/g) was reduced compared with the isolated cholesterol ester from each xanthoma (1.1±0.01 cal/g). There was a large irreversible collagen denaturation endotherm (peak temperature = 67°C; enthalpy 9.9 cal/g collagen) that corresponded to the tissue shrinkage noted by microscopy. After the collagen denaturation, the sample displayed double-peaked reversible liquid crystalline transitions of cholesterol ester, of enthalpy 1.18±0.1 cal/g, that were identical to transitions of isolated cholesterol ester.

Fibers dissected from xanthomata were examined by X-ray diffraction at temperatures below and above the cholesterol ester transition. At 20°C there was a weakly oriented equatorial reflection of Bragg spacing 36Å, which corresponded to the smectic phase of cholesterol ester, and a series of oriented collagen reflections. At 42°C the cholesterol ester reflection disappeared. Stretched fibers examined at 10°C showed good orientation of collagen and cholesterol ester reflections and, in addition, meridional spacings which indicated oriented crystallization of cholesterol ester. These studies suggest that a major component of tendon xanthomata is extracellular cholesterol ester which displays altered melting and molecular orientation as a result of an interaction with collagen. At xanthoma temperatures, the cholesterol ester is in a smectic liquid crystalline state, probably layered between collagen fibrils, with the long axis of the cholesterol ester molecules perpendicular to the axis of the collagen fiber. Such collagen-cholesterol ester interactions may favor the extracellular deposition of cholesterol ester derived either from intracellular sources or directly from plasma lipoproteins.

INTRODUCTION

Tendon xanthomata are deposits of lipid and connective tissue commonly found in patients with familial hypercholesterolemia. They display slow growth and regression, tending to parallel the degree of hyperlipidemia. These deposits show some similarities to atherosclerotic plaques, both chemically and structurally. Thus, cholesterol and cholesterol ester are their main lipid components (1, 2) and they contain large amounts of connective tissue and clusters of cells filled with lipid droplets (3) which resemble the foam cells of fatty streaks. Xanthomata probably do not synthesize significant quantities of cholesterol but derive this lipid from the plasma (4). After intravenous injec-

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Alan Tall was a fellow of the Medical Foundation Inc. Boston, Massachusetts during the course of this work. His current address is Department of Medicine, College of Physicians & Surgeons of Columbia University, The Presbyterian Hospital, New York 10032.

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tion of radioactive cholesterol, there is a gradual rise in xanthoma cholesterol specific activity which indicates slow uptake or exchange of cholesterol with the plasma compartment (5, 6).

Recently, the physical state of lipids in atherosclerotic tissue (7–9), in the spleen from a patient with Tangier disease (10), and in the plasma lipoproteins (11–13) has been investigated with techniques such as polarized light microscopy, scanning calorimetry, and X-ray diffraction. These procedures permit fresh, whole tissue to be examined, and give information about the structure and interactions of lipid under physiological conditions.

With similar techniques we have now undertaken a study of tendon xanthomata, in an attempt to define the structure of the lipid deposits and possible interactions with other tissue components.

METHODS

Patients. Three patients with severe, familial hypercholesterolemia had tendon xanthomata removed because the xanthomata were causing discomfort and had failed to respond to medical treatment.

Patient A was a 36-yr-old white woman who had developed tendon xanthomata at age 13 yr and had experienced an acute myocardial infarction at age 19 yr. During the previous 10 yr her plasma cholesterol was 300–400 mg/100 ml, with low density lipoprotein (LDL)[1] cholesterol 250–280 mg/100 ml, very low density lipoprotein (VLDL) cholesterol 20–30 mg/100 ml, and high density lipoprotein (HDL) cholesterol about 25 mg/100 ml. At different times she had been treated with nicotinic acid, cholestyramine, neomycin, and β-sitosterol, and had undergone ileal by-pass surgery at age 24 yr. Although partially successful, none of these treatments had reduced her plasma cholesterol to below 300 mg/100 ml. Tendon xanthomata were removed from both elbows and from the extensor tendon of her right fourth finger.

Patient B was a 30-yr-old black man with a 7-yr history of tendon xanthomatosis and hyperlipidemia. During this period his total plasma cholesterol was 300–400 mg/100 ml, with LDL cholesterol 240–290 mg/100 ml, VLDL cholesterol 25–40 mg/100 ml, and HDL cholesterol 25–35 mg/100 ml. He had never followed dietary or drug treatment. A xanthoma was removed from his right elbow.

Patient C was a 56-yr-old white woman with hyperlipidemia and tendon xanthomatosis of at least 15 yr duration. During the previous 6 yr her plasma cholesterol was 250–350 mg/100 ml with normal triglyceride, LDL cholesterol 190–230, VLDL cholesterol 40–60, and HDL cholesterol 35–40 mg/100 ml. She had not adhered to dietary or drug treatment. Tendon xanthomata were removed from the extensor tendon of her right second finger and from her left patellar tendon. In all cases the xanthomata were wrapped in saline-soaked gauze and examined by different techniques within 2–3 h after surgical removal. The samples were not cooled below 25°C and, except for specimens examined by standard light and electron microscopy, were analyzed without fixation.

Bovine tendon collagen was purchasd from Sigma Chemical Co., St. Louis, Mo.

[1]*Abbreviations used in this paper*: CE, cholesterol ester(s); HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

Chemical methods

Thin-layer chromatography. Lipids from homogenates of xanthomata were doubly extracted in 10 vol of chloroform: methanol (2:1 vol/vol), after which a Folch procedure (14) was carried out. Quantitative thin-layer chromatography was used to measure the free cholesterol, fatty acid, triglyceride, cholesterol ester, lysolecithin, sphingomyelin, and lecithin according to the method of Downing (15), as modified by Katz et al. (8). Cholesterol ester was isolated from the xanthoma lipids by preparative thin layer chromatography, with a hexane:ether (94:6 vol/vol) solvent system. Cholesterol ester spots were scraped and the silica removed by eluting the lipid with chloroform:methanol (2:1 vol/vol) through a sintered glass funnel.

Gas-liquid chromatography. Cholesterol esters were hydrolyzed in 2% alcoholic KOH, according to the method of Albrink (16) as modified by Smith and Slater (17).

Amino acid analysis. The delipidated xanthomata were hydrolyzed with 6 N HCl in sealed glass vials, under N_2 at 110°C, for 24 h, and amino acid composition was determined with a Technicon amino acid AutoAnalyzer (Technicon Instruments Corp., Tarrytown, N. Y.), according to the method of Hamilton (18). Hydroxyproline content of the hydrolysates was also determined by the method of Bergmann and Loxley (19).

Light and electron microscopy. For preparation of thin section electron micrographs, fresh samples were fixed in glutaraldehyde/paraformaldehyde within 10 min of excision from the patients, washed in Na cacodylate, fixed in OsO_4, washed in distilled water, dehydrated with acetone, embedded in Epon (Shell Chemical Co., Houston, Tex.), and sectioned with a glass knife (20). Sections were stained with Pb citrate and post-stained with uranyl acetate (21). Sections were examined with an AEI-6B electron microscope (AEI Scientific Apparatus Inc., Elmsford, N. Y.) calibrated with a catalase standard. All samples were also sectioned and stained with hematoxylin and eosin for conventional light microscopy.

Homogenization experiments. Xanthoma was cut into small pieces and homogenized in 0.15 M NaCl, with a VirTis homogenizer (VirTis Co., Inc., Gardiner, N. Y.) at a setting of 5,000 rpm for about 2 min. The homogenates were centrifuged for 1 h at 40,000 rpm in a Beckman 40.3 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), and the top, middle, and bottom 2 ml of each tube were analyzed for lipid.

Physical methods

Polarized light microscopy. A Zeiss standard NL microscope (Carl Zeiss, Inc., New York) fitted with a heating and cooling stage was used to identify the physical states of lipids in fresh, minced xanthomata. Samples were initially examined at 25°C and then heated or cooled at 2–3°C/min. Liquid crystalline cholesterol ester[2] was identified from its characteristic reversible liquid crystalline transitions between 30 and 40°C (22). The smectic phase was identified from its positive sign of birefringence and the cholesteric phase from its negative

[2]In the smectic liquid crystalline state there is a two-dimensional ordering of CE molecules into layers 36 Å apart, with the long axes of the CE molecules aligned. In the nematic liquid crystalline state, the long axes of the CE molecules are aligned but there is no layering of molecules. The cholesteric is a particular form of the nematic state, in which successive planes of CE molecules are aligned at slightly different angles, so that the planes form a screw or twist, of periodicity ≅ 4,000 Å.

sign. Cholesterol monohydrate was identified as plate crystals with a characteristic angle of 79° (8).

Differential scanning calorimetry. Pieces of fresh xanthoma were minced with a scalpel on a glass slide, transferred into 75-μl stainless steel pans, and examined with a Perkin-Elmer DSC-2 differential scanning calorimeter (Perkin-Elmer Corp., Norwalk, Conn.). Samples were heated at 5°C/min and cooled at 5, 10, or 20°C/min. The area enclosed by the transition endotherm and the base line, in conjunction with the mass of material in the sample pan, the instrumental sensitivity, and a calibration factor was used to calculate the transition enthalpy. Enthalpy and temperature were calibrated as described previously (23). Each sample was scanned a number of times in the temperature range of interest and multiple samples (three to five) were examined from each specimen.

X-ray diffraction. 1- to 3-cm fibers were dissected with fine scissors from the cut surface of xanthomata. In some instances unstretched fibers were placed in sealed Lindeman glass tubes (Lindeman Corp., Indianapolis, Ind.) and examined immediately after removal from the patient. However, better orientation of diffracted X rays was obtained with fibers that had been aligned by being stretched with a small weight (about 50 g) for about 24 h. The aligned fibers were examined while still under tension. X-ray diffraction studies were performed with nickel-filtered CuKα radiation from an Elliot GX6 rotating anode generator (Baird and Tatlock, London, England) and Elliot toroidal (Baird and Tatlock) or Franks mirror optics as described previously (11).

Results are expressed as mean±SEM. Significance of differences between means was determined by Student's *t* test.

RESULTS

Compositional analysis: lipid analysis. The tissue dry weights were 28.5–29.5% of the wet weights. Lipid constituted about 33% of the dry weight of the tissue (or 9.5±0.62% of the wet weight). The six xanthomata showed variable lipid composition (Table I) with a preponderance of cholesterol ester and cholesterol, and a paucity of phospholipid and triglyceride. Samples taken from different sites within the same xanthoma also showed some variability of lipid composition (Table I). A plot of the cholesterol, cholesterol ester, and phospholipid compositions on triangular coordinates (8) predicted the existence of separate cholesterol ester and phospholipid liquid crystalline phases in patients A and B and, in addition, a cholesterol crystal phase in patient C.

Cholesterol ester fatty acid analysis. To obtain enough material for analysis of fatty acid composition, samples were pooled from the different xanthomata in each patient. The cholesterol ester fatty acid composition was fairly uniform, and showed a relatively high content of saturated and monounsaturated fatty acids, and a low content of cholesterol linoleate compared, for example, to plasma LDL (11) or atherosclerotic fibrous plaques (17) (Table II).

Amino acid analysis. The material left after extraction of lipid from the xanthomas was hydrolyzed in HCl and subjected to amino acid analysis (Table III). Amino acids constituted 58±3.4% of the chloroform: methanol insoluble residue, or 39% of the dry weight. The amino acid composition was notable for a high content of hydroxyproline (7%) and glycine (25%), which indicates that collagen was the major protein constituent of the xanthomas. Assuming an 11% content of hydroxyproline in the tendon xanthoma collagen (based on the hydroxyproline content of tendon collagen), collagen constituted 36±1.5% of the nonlipid content of the xanthomas, or 24% of the dry weight. Direct analysis for hydroxyproline, with the method of Bergmann and Loxley (19) gave a value of 38.5±1.8%.

TABLE I
*Lipid Composition (%) of Xanthomata**

Patient	Site	Cholesterol	Triglyceride	CE	Lysolecithin	Sphingomyelin	Lecithin
A	Right elbow	14	1	70.8	0	4.3	9.5
		11	0.2	74.8	1	5.2	8.1
	Left elbow	11	0.2	79	0.5	4.3	5
		9	1	75	1.5	4.3	9
	Finger	16.1	—	71.2	—	4.8	7.8
B	Finger	7.1	3.0	80.5	0.25	3.5	4
		9.9	1.8	80.8	0.35	3.7	5.0
C	Knee	42	6.5	38	2.5	4.5	8
		34	5.4	46	1.3	5.5	7.3
	Finger	50	8	26	—	4.4	11
		22	7.4	46		4.4	19

* Lipids were analysed by double Folch (14) extraction and quantitative thin-layer chromatography (15). Results of duplicate samplings from different sites in the xanthomata are shown.

TABLE II
CE Fatty Acids (%) of Xanthomata

Patient	14:0	16:0	16:1	18:0	18:1	18:2	18:3(ω3)	20:2	20:3(ω9)	20:4	22:4	22:5(ω6)	22:5(ω3)	22:6(ω3)	other
A	0.8	9.0	6.5	2.1	50.9	19.2	1.4	1.6	2.6	3.2	0.9	0.5	0.3	0.4	0.7
B	0.8	7.3	6.8	1.0	50.4	17.6	2.0	2.4	3.2	4.0	1.3	0.5	0.5	0.4	1.8
C	0.5	11.7	0.7	0.8	42.3	23.3	1.7	2.0	3.8	6.2	2.1	0.6	0.0	0.8	2.2

Compared with collagen, the total amino acid content of the xanthomas was slightly enriched in acidic amino acids.

Centrifugation of homogenized xanthoma. When homogenized xanthoma was subjected to preparative ultracentrifugation, about 50% of the lipid formed a floating skin while 50% remained in the pellet. The composition of the lipid skin was cholesterol 3.9%, triglyceride 0.1%, cholesterol ester 93.8%, sphingomyelin 1.3%, and lecithin 0.9%; while the pellet contained cholesterol 13.6%, cholesterol ester 74.1%, lysolecithin 0.4%, sphingomyelin 5.2%, and lecithin 6.8%. The amount of cholesterol ester liberated from the tissue depended upon the time and vigor of homogenization. Thus, when the pellet formed from a first centrifugation was homogenized and centrifuged a second time, it liberated an equal proportion of its cholesterol ester. These results suggest that the homogenization procedure per se liberated cholesterol ester from the tissue.

Light microscopy. Sections stained with haematoxylin and eosin showed a few clusters of cells with foamy cytoplasm which resembled foam cells (the cell membranes represent the phospholipid liquid crystals predicted from lipid composition). In most areas of the tissue the cells were interspersed with thick bands of connective tissue.

Electron microscopy. Two of the three xanthomas from patient A, and the xanthoma from patient B, were examined by thin section electron microscopy. The principal constituent of all the specimens was bundles of fibrillar material (Fig. 1), which at high magnification was identified as collagen from its characteristic 620±12 Å periodicity (n = 13 micrographs). The mean diameter of the collagen fibrils was 455±20 Å, and although the space between fibrils ranged between 0 and 2,000 Å, the average distance was about 250 Å. All sections were remarkable for a paucity of large lipid droplets or cellular elements.

Polarized light microscopy. Examination of pieces of minced tissue at 25°C showed occasional smectic liquid crystals of free cholesterol ester and, also, in patient C, crystals of free cholesterol. The amorphous bulk of the tissue showed a diffuse background birefringence, which underwent no changes when cooled from 25°C to 10°C or subsequent heating to 60°C. When heated further, there was an abrupt shrinkage of the tissue, associated with separation of an oily phase, most evident at about 70°C. When cooled to 20°C, the oily material was identified as cholesterol ester which displayed characteristic reversible liquid crystalline transitions (22). In all three xanthomata of patient A, the smectic to cholesteric transitions occurred at 34°C, and the cholesteric to isotropic liquid transition at 37–38°C, while in patient B the respective temperatures were 37 and 41°C (Table IV). In patient C, both xanthomata showed a smectic-disordered transition at about 31°C, but no cholesteric phase could be seen, as expected from the high triglyceride content (Table I), because triglyceride abolishes the optical and calorimetric properties of the cholesteric phase (11).

Differential scanning calorimetry. Examination of fresh xanthoma by differential scanning calorimetry showed a reversible thermal transition between ≅30 and 45°C, of peak temperature 36–37°C, for patient A, 37–38°C for patient B (Fig. 2), and 31–32°C for patient C (Table IV). The enthalpy of this transition was 0.71±0.07 cal/g cholesterol ester (13 experiments on

TABLE III
*Amino Acid Composition (%) of Tendon Xanthomata**

Hydroxylysine	Lysine	Histidine	Arginine	Cysteic acid	Hydroxyproline	Aspartic acid	Threonine	Serine
0.54±0.02	4.3±0.26	1.2±0.06	5.5±0.09	0.57±0.05	7.12±0.4	6.9±0.26	3.1±0.71	4.4±0.24

Glutamic acid	Proline	Glycine	Alanine	Valine	Isoleucine	Leucine	Tyrosine	Phenylalanine
9.1±0.26	9.9±0.21	25.6±0.77	9.6±0.27	4.1±0.22	2.14±0.2	4.72±0.31	1.48±0.1	2±0.16

* Results shown are mean±SEM obtained from individual xanthomata (n = 6).

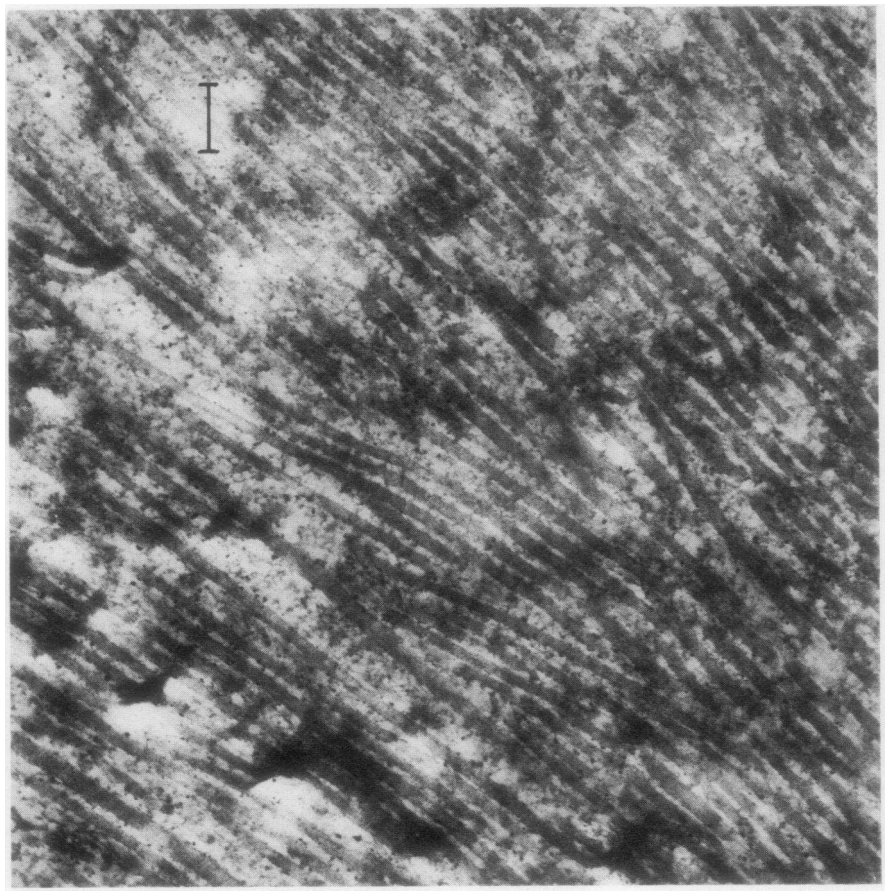

FIGURE 1 Thin section electron micrograph of xanthoma. Samples were fixed in glutaraldehyde/paraformaldehyde immediately after removal from the patients, sectioned, and stained as described in Methods. The fibrillar material was identified as collagen from its banding pattern of periodicity 620 Å. The bars indicate 2,000 Å.

six xanthomata). When heated further, there was a large irreversible endotherm at 67±0.6°C, of enthalpy 9.9±1.2 cal/g collagen. The mean onset and end temperatures (measured from the intersections of tangents to the transition with the base line) were 60 and 75°C. With subsequent cooling and heating, there were reversible smectic-cholesteric and cholesteric-liquid transitions of cholesterol ester at 32 and 37°C in patient

TABLE IV
*Temperature of CE Transitions**

Patient	Site	Intact xanthoma smectic-disordered	Xanthoma after thermal collagen denaturation		CE/Triglyceride	18.1/18.2
			Smectic-cholesteric	Cholesteric-liquid		
A	Right elbow	37	32–34	37	122	
	Left elbow	36	32–34	37	128	2.64‡
	Finger	37	33	37	∞	
B	Finger	38	34–37	41	33	2.88
C	Knee	31	-31-§		6.9	1.84‡
	Finger	32	-31-		4.5	

* Temperatures refer to the peak temperatures noted by differential scanning calorimetry and the median melting temperature of CE droplets observed by polarized light microscopy.
‡ Average value for patient.
§ In patient C, this was a single-peaked smectic-liquid transition.

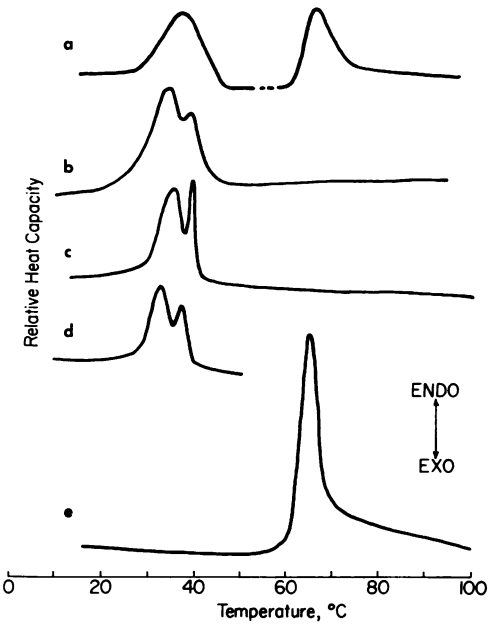

FIGURE 2 Differential scanning calorimetric heating curves of xanthoma specimens (*a*) fresh, minced xanthoma, (*b*) same specimen after being heated to 100°C, (*c*) CE isolated from xanthoma lipids, (*d*) lipid skin formed by preparative ultracentrifugation of homogenized xanthoma, (*e*) chloroform: methanol insoluble residue of xanthoma. All samples were heated at 5°C/min in hermetically sealed 75-μl sample pans. Endothermic (ENDO) transitions are represented by upward deflections of the base line, and exothermic (EXO) transitions by downward deflections. In (*a*), the scale sensitivity between 0 and 50°C is shown at 0.4 mcal/s, and between 55 and 100°C at 2 mcal/s (×⅕ sensitivity). Upon heating the intact xanthoma (*a*), there is a liquid crystalline transition of CE between 25–45°C, followed by an irreversible collagen denaturation. Upon subsequent heating (*b*), the CE transition displays an increased enthalpy. Results shown are for patient B.

A, 34 and 41°C in patient B (Fig. 2*b*), and a single transition of peak temperature 31°C in patient C. The total enthalpy of these liquid crystalline transitions of cholesterol ester of heat denatured xanthomata was 1.18±0.10 cal/g cholesterol ester which was significantly greater ($P < 0.01$) than that of intact xanthoma. Xanthoma kept for 1 mo at −20°C showed an initial endotherm of peak temperature ~38°C (patient B) and enthalpy 7 cal/g cholesterol ester, which indicates that the cholesterol ester had crystallized (12). When recooled, liquid crystal transitions were observed which were similar to those of the native xanthoma. When heated further, the collagen transition was observed which indicated that although storage at −20°C produced crystallization of cholesterol ester(s) (CE), this did not alter the structure of the collagen.

Pure CE isolated from the xanthoma of patient B showed liquid crystalline transitions (Fig. 2*c*) at identical temperatures to the heat-denatured, intact xanthoma (Fig. 2*b*). When examined by differential scanning calorimetry, the lipid skin obtained by preparative ultracentrifugation of homogenized xanthomata showed sharp transitions identical to unbound or unassociated CE (Fig. 2*d*). Delipidated material from the xanthoma showed a sharp, irreversible transition of peak temperature (68°C) and enthalpy ≅10 cal/g collagen, resembling the high temperature denaturation endotherm observed in the native tissue (Fig. 2*e*). A denaturation endotherm of identical temperature and enthalpy was obtained from a sample of bovine tendon collagen (not shown).

To determine the temperature dependence of the release of CE from intact xanthoma, tissue was systematically heated, cooled, and reheated to progressively higher temperatures (Fig. 3). Heating through the large, irreversible endotherm of peak temperature 67°C clearly resulted in a double-peaked endotherm of increased enthalpy (Fig. 3). When the increase in enthalpy of the CE transition was expressed as a function of temperature, a sigmoidal curve resulted (Fig. 4). The sigmoidal curve approximates the integrated form of the endotherm of peak temperature 67°C, and has the same midpoint temperature (67°C). Thus, the release of CE paralleled the thermal denaturation of collagen.

X-ray diffraction. The cut surface of the fresh xan-

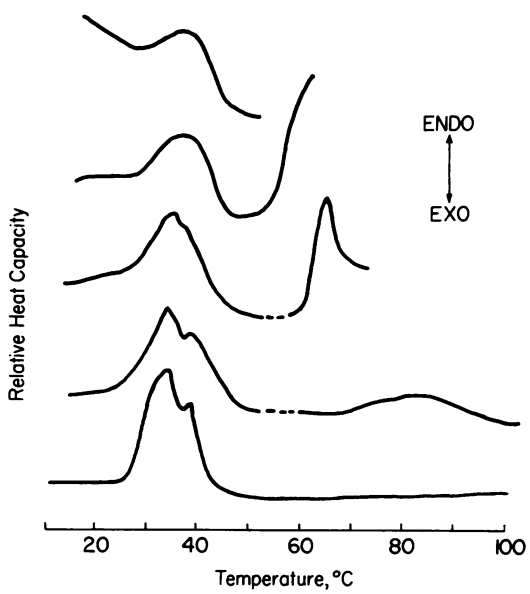

FIGURE 3 Differential scanning calorimetric heating curves of xanthoma. Xanthoma minced with a scalpel, was sealed in a 75-μl pan and then heated to 50°C, cooled to 0°, (cooling not shown), heated to 60°, cooled to 0°, heated to 70°, cooled to 0°, heated to 100°, cooled to 0°, and heated to 100°C. (Figure reads top to bottom.) The sensitivity was 0.4 mcal/s, except in sections initiated by dashed lines, where the sensitivity was 2 mcal/s. With heating to higher temperatures there is a progressive increase in the enthalpy of the transition between 25 and 45°C (CE), especially after the irreversible high temperature denaturation of peak temperature 67°C (collagen). ENDO and EXO show the direction of endothermic and exothermic transitions.

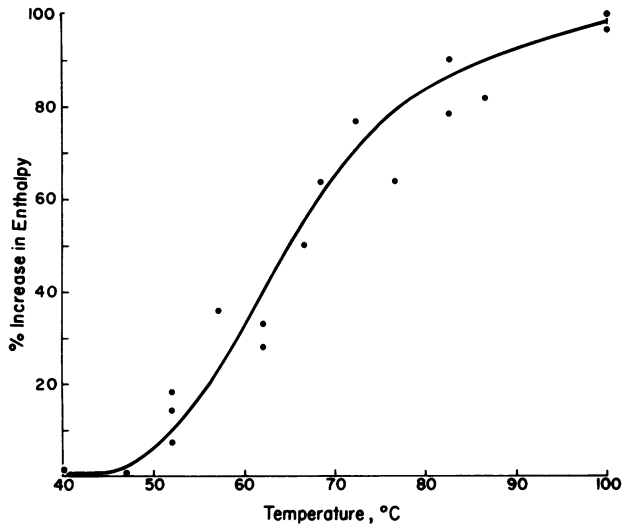

FIGURE 4 The increase in enthalpy of the xanthoma CE transition as a function of temperature. The xanthoma specimens (six experiments) were heated to progressively higher temperatures and the percent increase in the enthalpy of the CE liquid crystal melt measured as a function of the temperature of the previous heating run. In each experiment, the percent increase in enthalpy was determined from the increase in area of the CE transition after heating to a certain temperature ÷ total increase in area after heating to 100°C. The midpoint temperature of the increase in enthalpy (i.e., the temperature at which 50% of the increase occurred) corresponded to the peak or midpoint temperature of collagen denaturation (68°C), which suggests that the liberation of CE was a result of denaturation of collagen.

thoma consisted of a network of fibers embedded in a yellow matrix. X-ray diffraction performed on randomly selected, unoriented pieces of xanthoma immediately after removal from the patient showed a sharp diffraction fringe which corresponded to a Bragg spacing of 36 Å and diffuse fringes at about 15 Å and 4.8 Å. There were no short spacings of crystalline CE. The 36-Å spacing, characteristic of the smectic phase of CE, disappeared when heated to 45°C, while the diffuse fringes persisted. The 15-Å spacing is part of the diffraction pattern of collagen (24, 25). To obtain greater resolution, X-ray diffraction was performed on oriented fibers.

Partially oriented, unstretched fibers, examined below the CE transition (e.g., at 10°C) immediately after removal from the patients, showed a weakly oriented, 36-Å equatorial reflection arising from smectic liquid crystalline CE, and also a weakly oriented equatorial reflection at 15 Å arising from the collagen (Fig. 5*a*). To improve the alignment of collagen, fibers were stretched and examined while under tension. Under these conditions well oriented equatorial reflections at 36 and 15 Å were observed (Figs. 5*b* and *c*). In addition, a series of meridional reflections were observed, identical in spacing to those of crystalline CE isolated from the xanthomata (Fig. 5*d*). Thus, oriented crystallization of CE occurred during the preparation of these specimens. When heated to temperatures above the calorimetric transition of CE (e.g., 42°C) all of the CE diffractions disappeared, while the equatorial collagen spacing persisted (Fig. 5*e*). Examination of the low angle diffraction pattern of oriented, stretched fibers at 10°C showed a series of sharp, meridional reflections (Fig. 5*f*) which proved to be the 4th and 6th to 11th orders of the 620-Å periodicity of the collagen fibril (Table V). All areas of the xanthomata examined by X-ray diffraction showed collagen and CE diffractions with the relative intensities paralleling the chemical composition. Furthermore, 10 different fiber preparations showed the same orientation of CE with respect to the collagen fiber axis. The same patterns were observed for different sites in the same sample. In the xanthomata from patient C, which showed cholesterol monohydrate crystals by microscopy, there were additional reflections arising from crystalline cholesterol (e.g., note the

FIGURE 5 X-ray diffraction of xanthoma fibers (*a*) unstretched fiber, 10°C (toroidal camera), (*b*) streched fiber, 10°C (toroidal camera), short exposure, (*c*) as in (*b*), but longer exposure, (*d*) purified xanthoma CE, at 10°C, toroidal camera, (*e*) stretched fiber, 42°C, toroidal camera, (*f*) stretched oriented fiber, 10°C, Franks camera (lower angle region). All specimens are shown with the fiber axis (meridional) parallel to the length of this page. In (*a*), there are weakly oriented equatorial reflections, which correspond to Bragg spacings of 36 and 15 Å, arising from smectic CE and collagen, respectively. The spotty equatorial reflections at 34 and 17 Å are a result of crystalline cholesterol monohydrate. In (*b*), the 36-Å equatorial reflection is seen as an intense fringe close to the center of the film. In addition, there are a series of meridional reflections arising from crystalline CE. In (*c*), the orientation of the 36-Å fringe is not seen because of overexposure, but the equatorial reflection from collagen at 15 Å can be more clearly seen. On the original film, a 2.8-Å meridional spacing arising from collagen can also be seen. All of the fringes of crystalline CE shown in (*d*) can be seen on the original films of (*b*) and (*c*). CE of the xanthoma fibers (*b* and *c*) melts when heated to 42°C, shown in (*e*), leaving only the collagen reflections (15-Å equatorial and 2.8-Å meridional—the latter is seen on the original films). In (*f*) is shown the low-angle meridional diffractions of collagen (orders of 630 Å) and a strong 36-Å equatorial reflection arising from CE. There is also a weak 34-Å reflection due to unesterified cholesterol. (*a*) is from patient C and (*b-f*) from patient B.

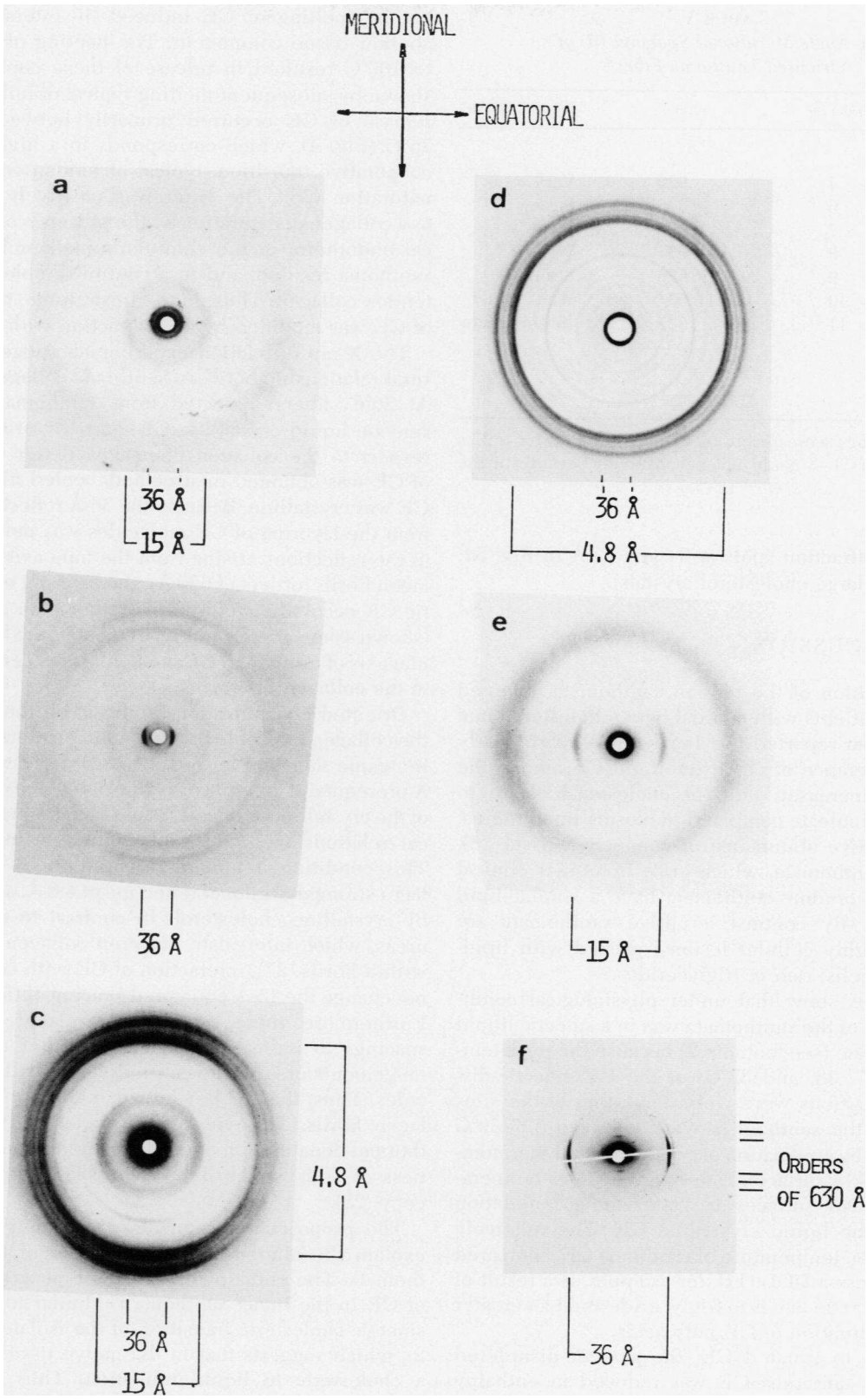
MERIDIONAL
EQUATORIAL
a
36 Å
15 Å
d
36 Å
4.8 Å
b
36 Å
e
15 Å
c
4.8 Å
36 Å
15 Å
f
ORDERS
OF 630 Å
36 Å

TABLE V
*Low Angle Meridional Spacings (d) of Oriented Xanthoma Fiber**

Spacing (d)	Order (n)	nX d	Relative intensity
Å		Å	
155	4	620	Very weak
106	6	636	Strong
91.2	7	638	Weak
78.9	8	631	Weak
69.6	9	626	Strong
63.5	10	634	Weak
57.8	11	635	Intermediate
		mean = 631 Å	

* These spacings were recorded with Franks' mirror optics at 10°C. Orders 1–3 could not be observed because of the backstop.

equatorial diffraction spots at 34 and 17 Å in Fig. 5*a*, arising from large cholesterol crystals).

DISCUSSION

The composition of the tendon xanthomata obtained from three patients with familial hypercholesterolemia resembles that reported previously (1–3), particularly the preponderance of cholesterol and CE among the lipids, the increased ratio of cholesterol oleate to cholesterol linoleate compared to plasma lipoproteins, and the relative abundance of connective tissue (3). Tuberous xanthomata, which arise in similar clinical situations to tendon xanthomata have a similar lipid composition. By contrast, eruptive xanthomata are transient, highly cellular lesions, packed with lipid-laden foam cells, rich in triglyceride.

Our studies show that under physiological conditions the CE of the xanthomata was in a smectic liquid crystalline state (see footnote 2) because the peak temperatures (37, 38, and 32°C) of the CE smectic-disordered transitions were well above that of the sites from which the xanthomata were removed (28–30°C as measured by application of a liquid crystal thermometer to the skin surface). Indeed, the cooler temperatures of extensor surfaces may favor the accumulation of the smectic liquid crystalline CE. The relatively high transition temperature of xanthoma CE, compared to human plasma LDL (11), for example, is a result of an increased ratio of CE to triglyceride and to a greater degree of saturation of CE fatty acids.

Compared to isolated CE, the smectic-disordered transition of xanthoma CE was reduced in enthalpy (from 1.1 to 0.7 cal/g CE), which indicates constraints on the melting of CE induced by interaction with another tissue component. The heating of the tissue to 100°C resulted in release of these constraints, as shown by subsequent melting typical of bulk CE. The release of CE occurred primarily between 55 and 75°C (Fig. 4), which corresponds to a high enthalpy cooperative transition, typical of tendon collagen denaturation (26). The latter was positively identified as a collagen denaturation by the presence of an identical endotherm in the chloroform:methanol insoluble xanthoma residue, and in a control sample of bovine tendon collagen. Thus, in the fresh tissue, the melting of CE was modified by an interaction with collagen.

The X-ray diffraction experiments suggest a structural relationship between xanthoma collagen and CE. At 20°C, fibers dissected from xanthomata showed smectic liquid crystalline CE, partially oriented with respect to the collagen fiber axis. Better orientation of CE was obtained in stretched, cooled fibers where CE was crystalline. Because the 36-Å reflection arising from the layering of CE molecules was perpendicular to the reflections arising from the long axis of the collagen fibrils (orders of 620 Å), the layering of the smectic CE occurs between fibrils rather than along them (shown schematically in Fig. 6). That is, the long molecular axis of individual CE molecules lies perpendicular to the collagen fiber axis.

Oriented crystallization (epitaxis) on the surface of the collagen molecule has been demonstrated for some inorganic salts and for organic urea derivates (24, 27). A prerequisite for epitaxis is that one of the dimensions of the crystalline-unit cell should bear a simple numerical relationship to the collagen layer line at 9.4 Å. This condition is apparently fulfilled by CE which has a strong meridional reflection at 4.8 Å, and perhaps by crystalline cholesterol. In contrast to the organic ureas, which intercalate between collagen molecules within fibrils (27), interaction of CE with collagen did not change the 15-Å equatorial spacing of the collagen. Furthermore, there were no lower angle equatorial spacings to indicate hexagonal or other ordered arrangements of CE between individual collagen molecules. Thus, the CE lies between and not within collagen fibrils. This view is supported by the normal thermal denaturation of collagen and the normal thickness of collagen fibrils as seen by electron microscopy (25).

The proposed collagen/CE structure (Fig. 6) may explain the altered melting behavior of CE of xanthomata. The enthalpy of the single-peaked transition of CE in the intact xanthoma is similar to that of the smectic-cholesteric transition of the isolated CE (Fig. 2), which suggests that in the native tissue, CE lacks a cholesteric to liquid transition. Thus, above the transition, CE may be held in a cholesteric-like or

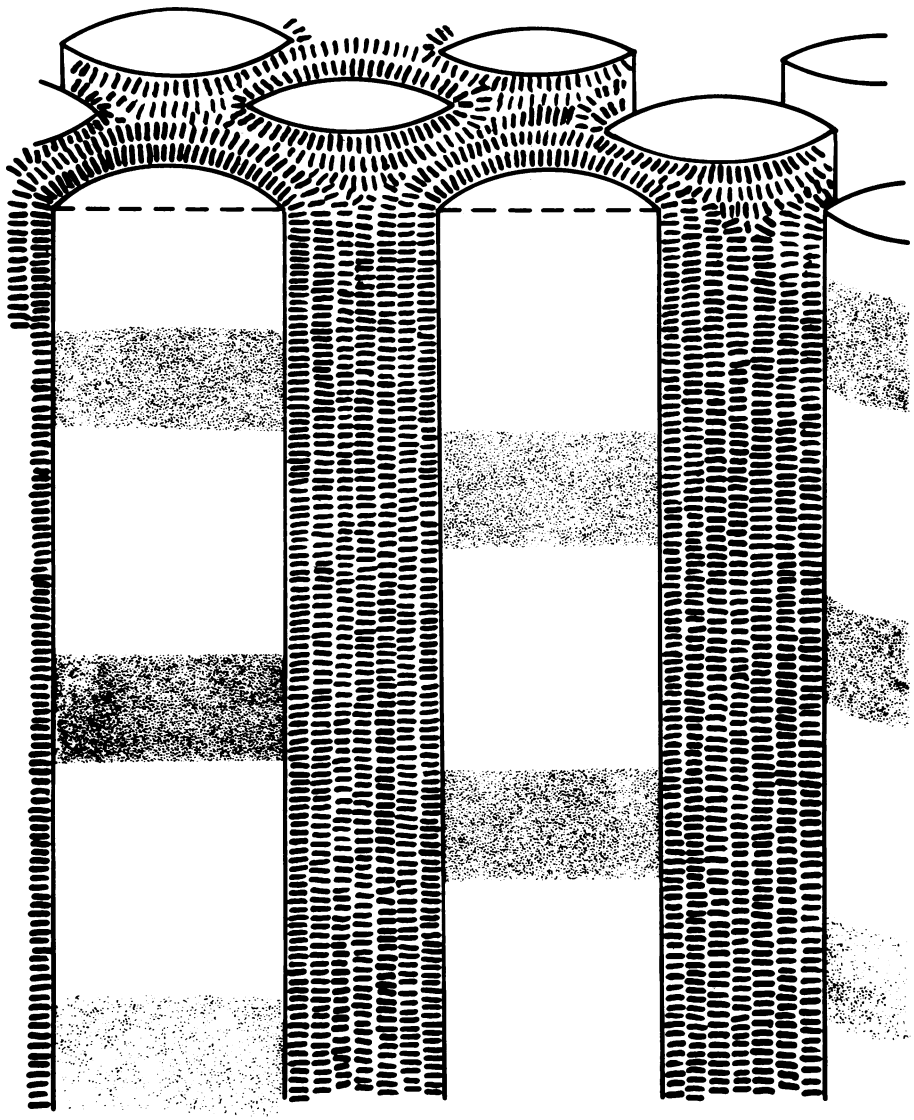

FIGURE 6 Model for CE and collagen interaction. CE is shown as —and collagen as crossbanded cylinder.

nematic state by the collagen molecule, with loss of layering between CE molecules but a persistent one-dimensional alignment of their long molecular axes.

It is unlikely that CE of xanthomata exists within intact LDL because the chemical composition, the low phospholipid content, and the high cholesterol oleate:linoleate ratio are unlike that of plasma LDL. Furthermore, xanthomata show partially oriented CE X-ray diffractions whereas LDL gives only unoriented CE reflections (11). Although we have identified an interaction between collagen and CE, we cannot exclude possible interactions of collagen or CE with other unidentified tissue components, for example, cholestanol esters or mucopolysaccharides.

That cholesterol and CE probably exist between and not within collagen fibrils suggests that the lipids are deposited after secretion of collagen from cells. Procollagen molecules are secreted from cells and assembled into fibrils subsequent to the action of extracellular enzymes which cleave off an NH_2-terminus peptide (28). CE could be deposited onto collagen directly from LDL, perhaps because of an affinity of the hydrophobic surface of the collagen fibril for CE. Alternatively, LDL CE may be taken up, hydrolyzed, and reesterified by cells which eventually die and liberate CE into the extracellular space. The latter process is suggested by the altered fatty acid composition of xanthoma CE, compared to LDL (Table II).

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